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BONE MARROW CELLULARITY IN POSTIRRADIATED RATS

ARMED FORCES RADIOBIOLOGY RESEARCH INSTITUTE
Defense Atomic Support Agency
Bethesda, Maryland

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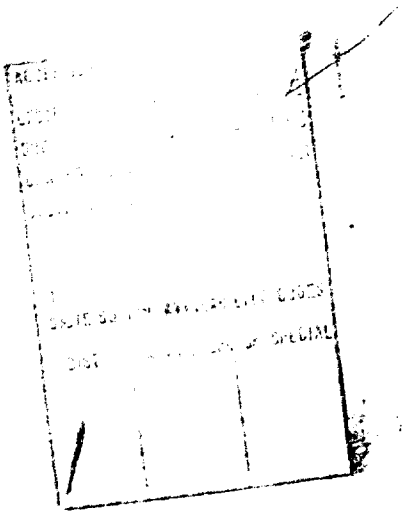

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BONE MARROW CELLULARITY IN POSTIRRADIATED RATS

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TABLE OF CONTENTS

LIST OF FIGURES

FOREWORD
(Nontechnical summary)

When man is exposed to large amounts of ionizing radiation, many of his tissues may be affected. One of the important tissues affected is the hematopoietic tissue (e.g., bone marrow) which is concerned with the development of blood cells. The marrow is different from the other tissues of the body in many ways, two of which are (1) it is a heterogeneous population of cells, and (2) the cells are actively dividing and differentiating in a process of changing from premature cells with no specific function to cells which will have a definite function in the blood and other body tissue. In an effort to better understand the effect of radiation in man, an analysis was made of rat bone marrow following selected doses of radiation when the cells in the marrow undergo changes associated with radiation damage and recovery. The different types of cells exhibit different radiation sensitivities but the response of each type could be correlated with the radiation dose. The objectives of this study included: (1) correlation of the difference in sensitivity of the cells with their division rate (reproducibility); and (2) determination of how related blood precursor cells influence the recovery of each cell type.

The rats were exposed to two radiation sources (x rays and mixed gamma-neutron radiation) and sacrificed at intervals for the purpose of determining the changes in numbers of cells in the marrow. The results indicate that the difference in the response (sensitivity) of the individual types of cells is not directly related to their division rate. The erythrocytic cells were the first to recover. This indicates that no other observed cell type could be responsible for its recovery unless it is

possible for (1) lymphocytic cells to differentiate into the erythrocytic cell compartment during lymphocytic cell depression, or (2) lymphocytic cells to function as trephocytes (giving up nutrients to newly forming erythrocytic cells through their death). The results, however, may be in contradiction with the belief that the lymphocytic cell functions as a special cell giving rise to the other marrow cell types. These observations call for a reevaluation of the lymphocyte stem-cell hypothesis.

ABSTRACT

The effect of sublethal doses of radiation on individual types of cells concerned with the development of blood cells has been studied in rat bone marrow. Observations were made for 40 days following exposures to x radiation and mixed gamma-neutron radiation. The changes associated with damage and recovery in the marrow were studied. The involved cells exhibited a difference in sensitivity in their response to radiation which could be correlated with radiation dose. The specific objectives of this study were (1) to determine to what extent cell turnover (reproduction) affects the difference in radiation sensitivity of the individual cell types, and (2) to investigate the relationship which exists among the cell types during the damage and recovery period.

Six hundred and seventy-two rats were exposed to either of the two radiation sources and sacrificed at periodic intervals following exposure for the purpose of determining the change in numbers of cells in the marrow. The results indicate that the difference in the response of the individual types of cells is not directly related to their turnover rate. The turnover rate for the myelocytic cells is greater than the turnover rate for the erythrocytic cells, yet the erythrocytic precursor cells are more sensitive to radiation than the myelocytic precursor cells. The results also show that erythrocytic cells recover before myelocytic or lymphocytic cells. This recovery indicates that lymphocytic cells probably could not give rise to the other two cell types (function as a stem cell) through a process of differentiation or dedifferentiation. However, it may be possible that the lymphocytic cells could function as trephocytes.

I. INTRODUCTION

A significant advancement in the study of the effect of ionizing radiation on hematopoiesis has resulted from the introduction of quantitative techniques for the evaluation of cell population kinetics.[11,16,20] These techniques, as subsequently used by Harris and Kugler,[15] Kurnick and Nokay[17] and Adachi[1] to study marrow cellularity during the depression and recovery of sublethally irradiated animals, revealed characteristics of the cell population as a function of time after irradiation. Harris[12] has described three distinct phases (injury, regeneration and completion of regeneration) exhibited by the marrow of gamma irradiated guinea pigs, in which at different times postirradiation some cell types increased in number while others decreased, indicating a difference in the sensitivity of the different cell populations exposed to radiation. Harris et al.[14] later expanded on their studies in a more detailed examination of the regenerative phase in which the accumulation of specific cell types was noted in the marrow of guinea pigs exposed to 150 and 200 rads of gamma radiation. However, to what extent the cell picture is dependent on the radiation source and dose was not reported. Adachi[1] attempted to answer this question, particularly with relation to dose, by using x-ray exposures of 500, 700 and 1000 R in an investigation of the processes of injury and regeneration in total-body exposed rats.

The specific objective of the present study was to investigate the possibility that not only the dose but the type of irradiation as well, might have an effect on the overall cell picture during injury and recovery. The present study delineates these

phases by quantitatively analyzing the main cell types of the marrow of rats exposed to x rays and a mixed gamma-neutron radiation source. This analysis could, in addition, provide information on the relative biological effectiveness (RBE) of the sources used in this particular cell system. An attempt was also made to correlate normal rates of cell turnover to fundamental differences in radiation sensitivity of the cell types and to show what relationship exists among the bone marrow cell types of irradiated animals during the recovery phase. The results in this paper imply that bone marrow lymphocytes do not function as stem cells under these conditions.

II. METHODS AND MATERIALS

The animals used were 672 Fischer rats,* 6-8 weeks old, weighing approximately 200 grams. These rats were housed one to a cage before and after irradiation in temperature-humidity controlled rooms. Food and water were given ad libitum.

The animals were exposed to two sources of radiation, Maxitron (x rays) and AFRRI-TRIGA reactor (mixed gamma-neutron radiation). The physical characteristics of the x-ray machine were as follows: 250 kVp, 30 mA, animal midline distance 60 cm from the x-ray tube; filtered by 0.95 mm Cu and 1.2 mm Be; HVL-1.9 mm Cu. The radiation field, free-in-air, was uniform to ± 4 percent. The x-ray doses used were 100, 300 or 500 rads midline in air at a dose rate of 20 rads per minute. The animals exposed to mixed gamma-neutron radiation from the AFRRI-TRIGA reactor also received doses of 100, 300 or 500 rads at a dose rate of 20 rads per minute. Reactor neutron doses were based on the neutron kerma calculated from threshold foil data and reactor gamma doses were calculated from silver activated phosphate glass data.[8]

* Obtained from Microbiological Associates, Bethesda, Maryland

The animals were sacrificed at various intervals (6 hours to 28 days postirradiation) for the purpose of determining the quantitative changes in the marrow cells at specific times during an extended postirradiation period. The animals were first anesthetized with ether and then exsanguinated from the right ventricle. Red and white cell counts were obtained from the collected blood and smears were made for blood differential counts. Both femurs were removed and the marrow forced out into a saline solution. The marrow of one femur was used for total cell count determinations and the other for preparing smears for differential counts.

The bone marrow smears were made by smearing a marrow fragment between two cover slips or between a slide and cover slip. The thin smears were dried, stained with Wright's stain for 2 minutes, flooded with pH 7.0 buffer, counterstained with Giemsa stain for 10 minutes, washed with distilled water and dried.

The specific groups of bone marrow cells investigated were the blast forms, lymphocytic, myelocytic and erythrocytic cells. Bone marrow differential counts were made of the individual cell types in normal and irradiated animals. Harris and Burke[10] previously reported that bone marrow undergoes a considerable change in composition during the first 10 weeks of life. Therefore the control animals utilized were the same age as the experimental animals. The number of cells per mm^3 was determined as follows: a known volume of rat bone marrow was placed in a known volume of diluting fluid. Cell counts were made from aliquots of the cell suspension utilizing the Coulter counter and the hemocytometer. The results represent the total number of cells per unit volume of marrow. The number of the individual types of cells per unit volume was determined by counting 500 cells on the differential smear prepared from the

same animal, determining the fraction of each cell type present, and multiplying it by the total number per unit volume.

III. RESULTS

The quantitative changes of the cells investigated are shown in Figures 1-3. Figures 4 and 5 represent a comparative summary of the results in Figures 1-3. These results indicate that radiation sensitivity varied according to cell type and that a separate dose dependency existed for each type. The more sensitive cells (lymphocytic and erythrocytic) showed a significant depression at the first sampling period (6 hours postirradiation). The myelocytic cells were not significantly decreased until one to two days after exposure to the six doses used. The differences in the time of bone marrow repopulation among these cell types were even more dramatic. These results are presented below.

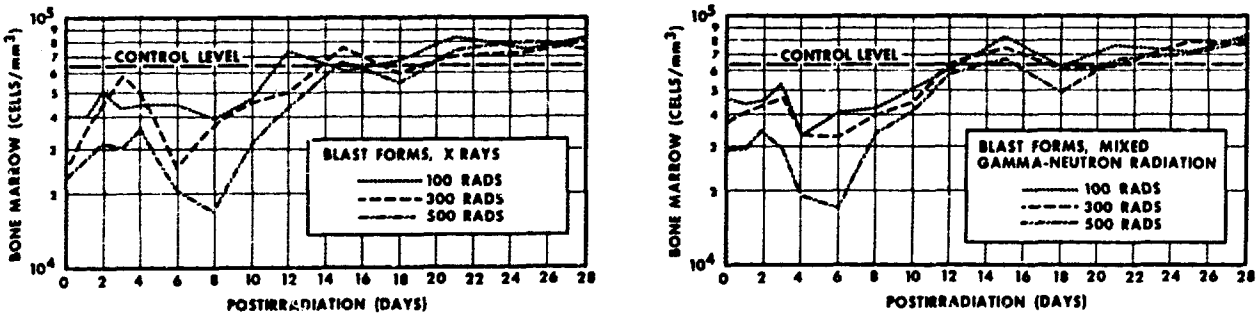

Figure 1. Quantitative changes in the blast forms of the bone marrow of Fischer rats exposed to whole-body doses of 100, 300 and 500 rads of x and mixed gamma-neutron radiation

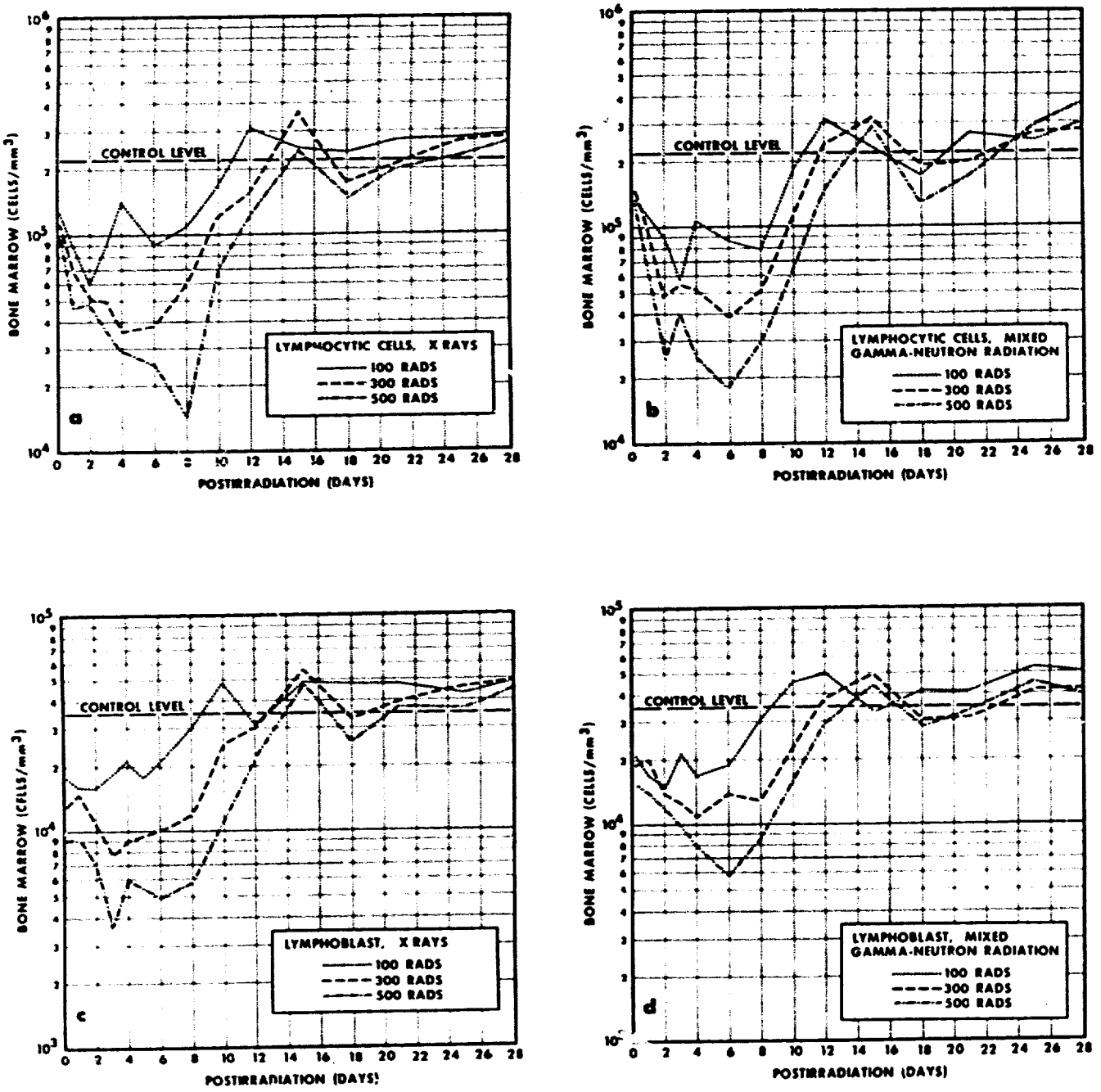

Figure 2. Quantitative changes in the lymphocytic cells (a and b) and the lymphoblast (c and d) of the bone marrow of Fischer rats exposed to whole-body doses of 100, 300 and 500 rads of x and mixed gamma-neutron radiation

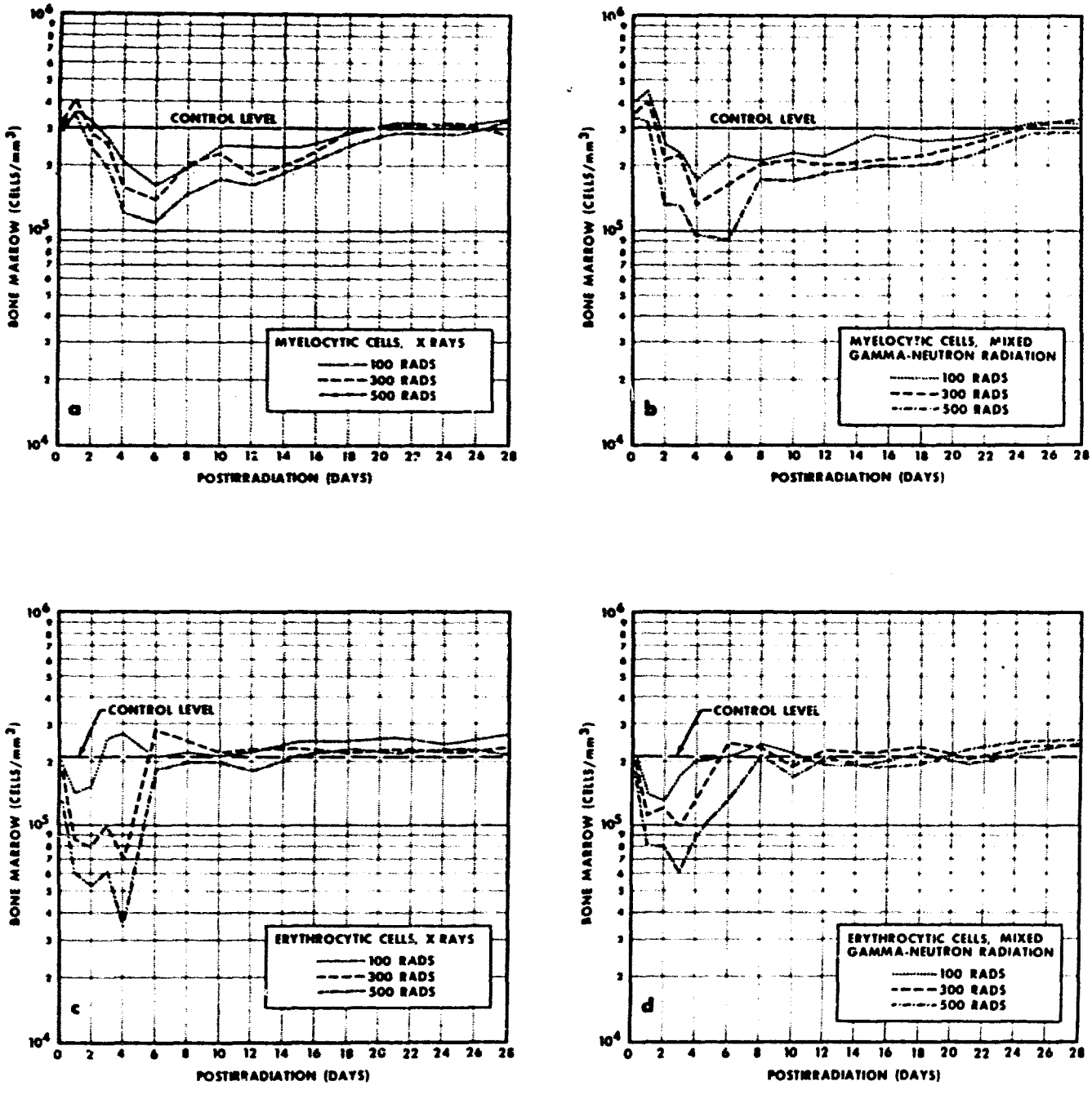

Figure 3. Quantitative changes in myelocytic cells (a and b) and erythrocytic cells (c and d) of the bone marrow of Fischer rats exposed to whole-body doses of 100, 300 and 500 rads of x and mixed gamma-neutron radiation

The blast forms are the earliest recognizable forms of the lymphocytic, myelocytic and erythrocytic cells. The concentration of blast forms in the bone marrow of control rats was ~6.5 x 10^4 cells per mm^3. The lymphocytic cells include the total lymphocyte population (i.e., the lymphoblast, mature, small, medium and large lymphocytes). The control level for these cells was ~2.3 x 10^5 cells per mm^3 and made up ~31.6 percent of the nucleated cell population. The lymphoblast (blast form of the lymphocytic cells) represented ~17.1 percent of the lymphocytic cells and numbered ~ 3.4 x 10^4 cells per mm^3 in the control animals (Figure 2). The control level for the myelocytic cells was ~2.9 x 10^5 cells per mm^3 (Figure 3). This constituted about 40 percent of the nucleated cells of the Fischer rat bone marrow. The erythrocytic cells represented all of the red blood cell precursors. The control level for these cells was ~2.1 x 10^5 cells per mm^3 (Figure 3) and constituted ~28.2 percent of the nucleated cells of the marrow.

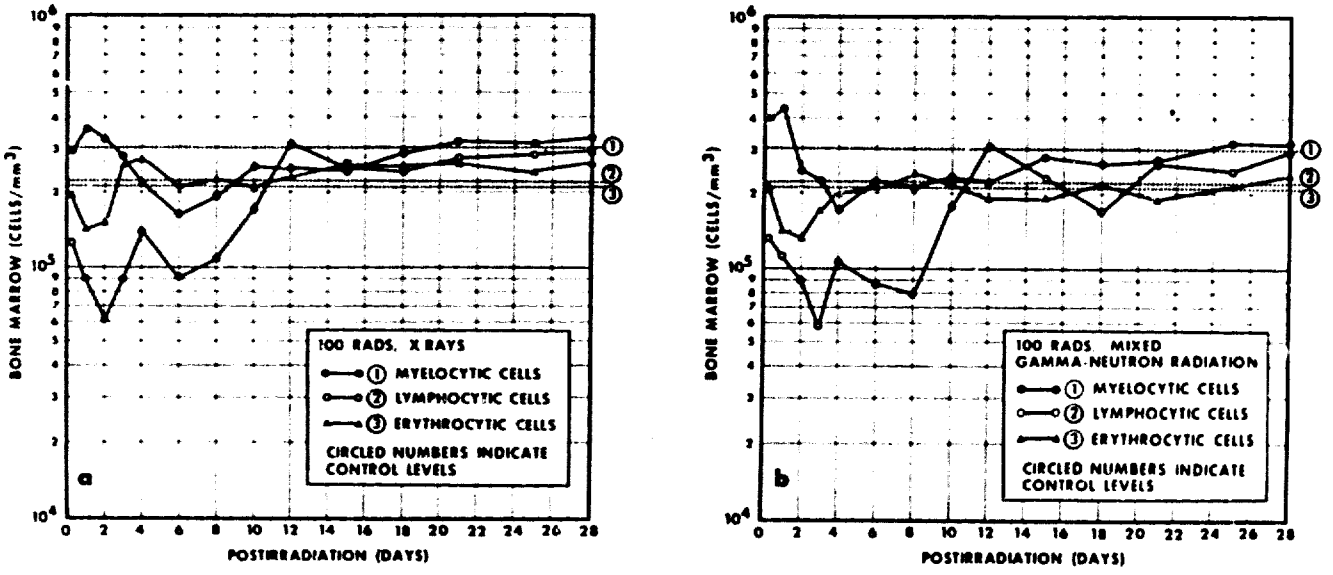

Figure 4. Comparison of quantitative changes in the myelocytic, lymphocytic and erythrocytic cells of the bone marrow of Fischer rats exposed to whole-body doses of 100 rads of (a) x radiation and (b) mixed gamma-neutron radiation

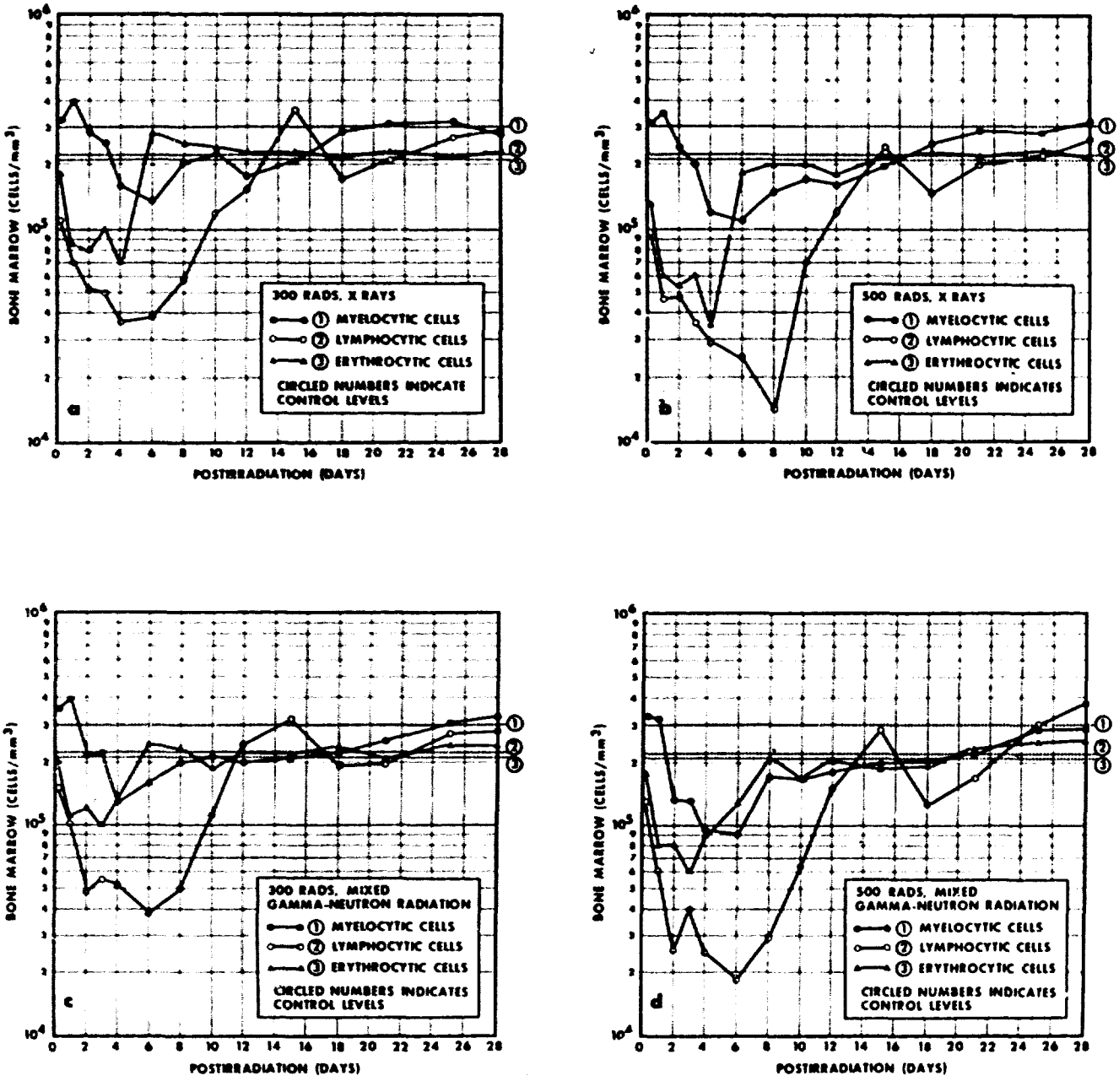

Figure 5. Comparison of quantitative changes in the myelocytic, lymphocytic and erythrocytic cells of the bone marrow of Fischer rats exposed to whole-body doses of (a) 300 rads and (b) 500 rads of x radiation, and (c) 300 rads and (d) 500 rads of mixed gamma-neutron radiation

The curves plotted for the number of all of the blast forms in the postirradiated animals can be seen in Figure 1. These curves indicate a significant depression ($p = 0.01$) in cell number during the first 6 hours after irradiation. The acute sensitivity of the lymphocytes to radiation is shown in Figure 2. This is in agreement with what others have found in the marrow of different species of animals after a sublethal dose of radiation. There is a dramatic drop in cell number in the marrow of the irradiated animals during the first 6 hours after exposure. The lymphoblast's quantitative response to radiation is very similar to the response of the mature lymphocytic cell population (Figure 2). Unlike the blast forms and lymphocytic cells, the myelocytic cells do not show a quantitative depression during the 1st day after exposure (Figure 3). The change which takes place is clearly an increase in myelocytic cell number in all of the irradiated animals with the exception of those exposed to 500 rads of mixed gamma-neutron radiation. The period of depression for the erythrocytic cells begins within 6 hours after exposure (Figure 3). The initial depression of the blast forms is followed by an increase in cell number reaching a peak on 2-4 days after exposure, depending on the dose used (Figure 1).

The depression in lymphocytic cell number continues to decrease and reaches its lowest level between day 2 and day 8 depending on the radiation dose (Figure 2). These depressions indicate a wide variation in the lymphocytic cell response depending on the radiation source and dose. The lymphocytic cells are at their lowest value as early as the 2nd day postirradiation in the animals exposed to 100 rads of either x rays or mixed gamma-neutron radiation. What can be described as an abortive rise can also

be seen in these results on day 4 followed by a second decrease in cell number. Animals exposed to higher doses have lower numerical lymphocytic cell values on later days. Results from x irradiated animals show a direct relationship between dose and decreased values of lymphocytic cells in terms of days after exposure.

The lymphoblasts are very susceptible to radiation as indicated by the depression phase of the radiation response curves. The decreased levels in cell number are directly related to the dose (Figure 2). The myelocytic cells had a comparatively gradual depression with lowest values at 4 to 6 days after exposure (Figure 3). The period of cell depression of the erythrocytic cells continues to significant depressions ($p = 0.01$) at days 3 and 4, except for the 100-rad dose when recovery was seen earlier.

The period of recovery for the blast forms begins immediately after the second period of greater cell depression (6-8 days postirradiation) and continues until the cells are again in the range of the control number 12-14 days after exposure. The time at which the cells return to normal is also dose dependent (e.g., the cells of rats exposed to lower doses recover earlier). The lymphocytic cells return rapidly to levels of the control numbers (Figure 2). The return begins on day 6 for the lymphocytic cells of rats exposed to 100 and 300 rads of x radiation and 300 and 500 rads for mixed gamma-neutron radiation. The lymphocytic cells of rats exposed to 500 rads of x radiation and 100 rads of mixed gamma-neutron radiation begin their regeneration on day 8. The completion of regeneration is marked by an "overshoot" at which time the cell concentration goes beyond control levels. It does not, however, remain significantly above control levels. There is another depression in cell concentration

on the 18th day after exposure which cannot be explained as a fluctuation in cell number because it characterized cell response of animals exposed to the two radiation sources at the three dose levels.

The myelocytic cell recovery begins on the 6th postirradiation day and extends over a long period of time. The cell counts return to control levels on 18-24 days after irradiation (Figure 3). The differences in the time of completion of regeneration in the cells of animals exposed to the three doses vary to an insignificant degree. The erythrocytic cells of animals exposed to 100 rads of x radiation or mixed gamma-neutron radiation return rapidly to control levels within 2-1/2 to 4 days after exposures. These cells return to control levels between days 5 and 6 when the animals are exposed to high doses with the exception of animals exposed to 500 rads of mixed gamma-neutron radiation when the return occurs on about the 8th day after exposure.

The bone marrow cell values of animals exposed to a particular x-ray dose were compared to bone marrow cell values of animals exposed to a similar dose of mixed gamma-neutron radiation. The RBE of the radiation used did not show an overall significant difference.

IV. DISCUSSION

The quantitative analysis of the main cell groups in the marrow of rats exposed to x rays and mixed gamma-neutron rays leads to some interesting observations concerning the relationship between (1) the individual sensitivity of the cell groups, (2) cell turnover, and (3) the relative concentration of the erythrocytic, myelocytic and lymphocytic cells during the various phases. The fact that the erythrocytic and lymphocytic cells respond more rapidly to radiation than do the myelocytic cells was first

reported by Bloom and Jacobson in 1948.[3] Similarly, the accumulation of lymphocytes in regenerating bone marrow before the recovery of myelocytic and erythrocytic cells has also been known for some time.[5] More recently Adachi[1] has put the erythrocytic and myelocytic cells in their order of regeneration and reported that the regenerative phase of the erythrocytic cells occurs before the regenerative phase of the myelocytic cells.

The fundamental difference of radiation sensitivity, in general, rests in the ability of the cells to reproduce, i.e., actively reproducing cells are generally more radiosensitive than those reproducing more slowly.[2] Studies in synchronized population of HeLa cells[21] indicate that cells are most sensitive during the mitosis, late prophase or early S (DNA synthesis) phase. The work of Till and McCulloch[23] indicates that the radiation sensitivity of the bone marrow cells may relate to both of the above observations.

Studies on the life span and the renewal time of lymphocytic, myelocytic and erythrocytic cells present some difficulties. Nevertheless, they reveal the existence of distinct differences in the respective mean life spans and the generation time of their precursors. The life span of myelocytes is relatively short. The life span of erythrocytes is intermediate and that of lymphocytes is relatively long. Calculations of the generation (renewal) time for myelocytic and erythrocytic cell precursors have been made by Patt.[19] He estimates a renewal time for the immediate erythrocyte precursors of 77 hours in the rat. For the same developmental stage in rat myelocytic cells he gives an estimate of 39 hours. This observation indicates that the cell renewal time for the erythrocyte precursors is approximately twice as long as the

cell turnover time for the myelocytic cells. Mitotic indices of 0.65 and 1.3 percent for erythrocyte and myelocyte precursors respectively were obtained by Widner et al.[26] With this information, it is reasonable to assume that there would be a larger number of myelocyte precursors in division than erythrocyte precursors if the precursor pools for both are relatively equal. From the above observations, it is expected that the radiation sensitivity of the hemic cells might be related to their respective turnover rates. Unfortunately, this is not as simple as it might seem. The results presented in Figures 4 and 5 indicate that the lymphocytic and erythrocytic cells of the bone marrow in whole-body irradiated rats are more susceptible to radiation than the myelocytic cells.

The presence of a large number of erythrocytic cell precursors (late normoblasts) in the bone marrow of rats between 1 and 3 days after exposure to the doses used was noted in this experiment. This indicates that there is a curious phenomenon which exists among these cells. It appears to represent a delay in the rate of maturation[4] at the onset of irradiation followed by a return to normal maturation time. This delay is also reflected in the release of erythrocytes to the peripheral blood. Since the marrow is a fixed space, the reduction must be compensated for in some manner. According to Bond et al.[4] this is done initially by the dilation of the vascular sinusoids and later by hemorrhage and resultant extravascular red cells. This explanation does not, however, exclude the possibility that the increase in mature red cells in the marrow may be due in part to induced maturation of late erythrocytic cells.

A relationship between erythrocytic, myelocytic and lymphocytic cells in the bone marrow of irradiated animals during the recovery phase has also been indicated

by other investigators. According to Harris and co-workers[12-15] the presence of an unusually large number of cells resembling the lymphocyte at the early stage in final recovery of bone marrow of irradiated guinea pigs suggests a multipotential function for the lymphocytes. This is supported by a study on repopulation of bone marrow of irradiated mice by Kurnick and Nokay[17] in which evidence was found to show that the cell responsible for bone marrow repopulation and x irradiation protection resembles the small lymphocyte. Fliedner et al.[9] in a study of the fate of bone marrow cells in irradiated recipients concluded that the stem cell responsible for marrow repopulation must be the mononuclear cell. Thomas et al.[22] observed that during a very rapid recovery of marrow function of dogs given near-lethal doses of nitrogen mustard the sequence of development indicated that the stem cell was morphologically indistinguishable from the small "lymphoid" cell. Cudokowicz et al.[7] also found evidence in their studies which suggested that the small marrow lymphocyte may be a pluripotent hemopoietic cell. Trowell,[24] in a study on some properties of lymphocytes in vivo and in vitro, discussed the transformation of small lymphocytes to monocytes and macrophages and offered a schema to show the cell interrelationships. There is no attempt here to prove or disprove special properties attributable to lymphocytes during bone marrow recovery, but merely to point up some interesting observations indicated by this study. The regenerating marrow of the rats indicated a possible multipotential function for the lymphocytes with a somewhat different interpretation. An unusually large number of cells resembling the lymphocytes at the early stage in final recovery was not found. The results indicated that the erythrocytic precursor cells were first to return to normal levels, followed by the lymphocytes and then by the myelocytes.

These results may be in contrast with the above hypothesis that the lymphocytes function as a multipotential cell giving rise to other marrow cells during the recovery phase of the depleted bone marrow cell population by a transformation process. Figure 4 shows a return of the erythrocytic cells 7 days before the lymphocytes, i.e., the erythrocytic cells reach a normal level at day 6 after exposure and the lymphocytes complete regeneration on day 13. However, if the return of the lymphocytes is compared to the myelocytes, the results could be considered as evidence for the presence of lymphocytes in the marrow early enough and in sufficient numbers to act as a multipotential cell and to give rise to the myelocytes. This may come about through a differentiation or a dedifferentiation of these cells to the myelocytes. The results of the present study indicate that the myelocytes of rats exposed to 300 rads x radiation return to normal levels after the depression period on day 20 (Figure 3), which is considerably longer than regeneration of the lymphocytes in the same animal.

Loutit[18] suggested the possibility that lymphocytes may function in part as trephocytes giving up, upon their death, DNA and other nutrients to newly forming cells. Nutrients released from the necrotic lymphocyte could induce inhibited cells to divide. The present results do not rule out this possibility. Whitfield et al.[25] reported that increasing the sodium concentration of the medium of suspension cultures of rat bone marrow accelerated the entry of irradiated suspensions (75 and 100 R) into mitosis and reduced the duration of postirradiation mitotic delay.

The results of the present study appear to give support for the trephocyte hypothesis because initial erythrocytic cell recovery occurs between 4 to 6 days when the number of lymphocytes are at a minimum. It could be assumed that this would

also be the time of maximum lymphocytic destruction. However, it has been indicated[6] that lymphocytes undergo pyknosis, karyorrhexis and karyolysis within just a few hours after exposure and that after irradiation, disintegration of the lymphocytes can be seen within 3 hours in the thymus and lymph nodes. Furthermore, almost all of the debris disappears within 24 hours after irradiation.

It may be more logical to assume that the lymphocytes remain at low levels during regeneration of erythrocytic cells because of possible rapid differentiation into the other cell line. The possible function of lymphocytes in this capacity does not necessarily depend on large numbers of lymphocytes being present just before the onset of erythrocytic cell recovery. If during regeneration of the marrow, lymphocytes were truly stem cells, they might be differentiating so rapidly that their concentration would be very low.

The last possibility is of course that the lymphocytes are not stem cells, at least in the rat. Since it has been suggested that bone marrow lymphocytes could be stem cells in dogs,[22] mice[7] and guinea pigs,[12] this would represent a species difference. It suggests that such observations cannot be used as a generalization to all animals.

Our observations seem to indicate very clearly that the problem of stem cell identification is still open, and new and better experimental approaches are needed to solve it.

V. SUMMARY

Bone marrow cell depression and regeneration induced by radiation were studied in rats exposed to x and mixed gamma-neutron radiations. What extent the cell

renewal time has on radiation sensitivity in these cells and what relationship exists among the cell types were of primary importance. The results show that the difference in the response of the individual types of cells is not directly related to their turnover rate as would be expected. The characteristics of the recovery curves for the individual cell types indicate that the erythrocytic cells are the first group of cells to return to control levels in regenerating marrow. This observation calls for a reevaluation of the lymphocyte stem-cell hypothesis.

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13. ABSTRACT

The effect of sublethal doses of radiation on individual types of cells concerned with the development of blood cells has been studied in rat bone marrow. Observations were made for 40 days following exposures to x radiation and mixed gamma-neutron radiation. The changes associated with damage and recovery in the marrow were studied. The involved cells exhibited a difference in sensitivity in their response to radiation which could be correlated with radiation dose. The specific objectives of this study were (1) to determine to what extent cell turnover (reproduction) affects the difference in radiation sensitivity of the individual cell types, and (2) to investigate the relationship which exists among the cell types during the damage and recovery period.

Six hundred and seventy-two rats were exposed to either of the two radiation sources and sacrificed at periodic intervals following exposure for the purpose of determining the change in numbers of cells in the marrow, The results indicate that the difference in the response of the individual types of cells is not directly related to their turnover rate. The turnover rate for the myelocytic cells is greater than the turnover rate for the erythrocytic cells, yet the erythrocytic precursor cells are more sensitive to radiation than the myelocytic precursor cells. The results also show that erythrocytic cells recover before myelocytic or lymphocytic cells. This recovery indicates that lymphocytic cells probably could not give rise to the other two cell types (function as a stem cell) through a process of differentiation or dedifferentiation. However, it may be possible that the lymphocytic cells could function as trephocytes.

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14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
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radiation effects						
bone marrow						
rats						
cells						
lymphocytes						
erythrocytes						
myelocytes						
hematopoiesis						
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